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Wild-type *APC* predicts poor prognosis in microsatellite-stable proximal colon cancer

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Background: *APC* mutations (*APC*-mt) occur in ~70% of colorectal cancers (CRCs), but their relationship to prognosis is unclear.

Methods: *APC* prognostic value was evaluated in 746 stage I–IV CRC patients, stratifying for tumour location and microsatellite instability (MSI). Microarrays were used to identify a gene signature that could classify *APC* mutation status, and classifier ability to predict prognosis was examined in an independent cohort.

Results: Wild-type *APC* microsatellite stable (*APC*-wt/MSS) tumours from the proximal colon showed poorer overall and recurrence-free survival (OS, RFS) than *APC*-mt/MSS proximal, *APC*-wt/MSS distal and *APC*-mt/MSS distal tumours (OS HR$\geqslant$1.79, $P\leqslant$0.015; RFS HR$\geqslant$1.88, $P\leqslant$0.026). *APC* was a stronger prognostic indicator than *BRAF*, *KRAS*, *PIK3CA*, *TP53*, CpG island methylator phenotype or chromosomal instability status ($P\leqslant$0.036). Microarray analysis similarly revealed poorer survival in MSS proximal cancers with an *APC*-wt-like signature ($P=$0.019). *APC* status did not affect outcomes in MSI tumours. In a validation on 206 patients with proximal colon cancer, *APC*-wt-like signature MSS cases showed poorer survival than *APC*-mt-like signature MSS or MSI cases (OS HR$\geqslant$2.50, $P\leqslant$0.010; RFS HR$\geqslant$2.14, $P\leqslant$0.025). Poor prognosis *APC*-wt/MSS proximal tumours exhibited features of the sessile serrated neoplasia pathway ($P\leqslant$0.016).

Conclusions: *APC*-wt status is a marker of poor prognosis in MSS proximal colon cancer.

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Colorectal cancer (CRC) is a leading cause of cancer-related death in the Western world (Stewart and Wild, 2014). Despite advances in surgery and oncology, the average 5-year survival rate remains below 60%. Treatment decisions are primarily based on disease staging performed by imaging and tumour histopathological assessment. However, there exists substantial heterogeneity in prognosis within tumours of identical stage.

Approximately 70% of sporadic CRCs are initiated by biallelic inactivation of the *APC* tumour-suppressor gene, resulting in aberrant activation of WNT/β-catenin signalling (Christie *et al*, 2013). The majority of *APC*-mutated (*APC*-mt) cancers are thought to develop via the classic adenoma-carcinoma pathway, characterised by premalignant adenomatous polyps with tubular and/or villous architecture, and carcinomas associated with *TP53* mutation and chromosomal instability (CIN) (Fearon and Vogelstein, 1990). Another major pathway to CRC is the sessile serrated pathway, accounting for 15–20% of cases (Leggett and Whitehall, 2010; Snover, 2011; Bettington *et al*, 2013). This pathway is typified by precursor sessile serrated adenomas, wild-type *APC* (*APC*-wt), *BRAF* mutation, a CpG island methylator phenotype (CIMP-high), poor differentiation and mucinous histology, and approximately half of sessile serrated pathway cancers show late development of microsatellite instability (MSI). A further alternate pathway has been proposed, perhaps comprising 5–10% of CRC, which may arise from traditional serrated or tubulovillous adenomas and exhibits CIMP-low, *KRAS* and *PIK3CA* mutation and a microsatellite stable (MSS) genotype (Leggett and Whitehall, 2010; Day *et al*, 2013). Notably, these pathways show differential distributions throughout the large intestine, with the sessile serrated pathway more frequent in the proximal colon, and the classic pathway more common in the distal colon and rectum.

Individual molecular characteristics associated with the neoplasia pathways including CIN, MSI, CIMP and mutations in *KRAS*, *BRAF*, *PIK3CA* and *TP53* have all been evaluated as indicators of patient prognosis. Multiple studies have demonstrated good outcomes for early-stage cancers with MSI, and combined with data indicating that MSI cancers may not benefit from 5-FU-based chemotherapy, it has been proposed that moderate- and high-risk stage II patients exhibiting an MSI phenotype may forego adjuvant chemotherapy (Popat *et al*, 2005; Guastadisegni *et al*, 2010; Ng and Schrag, 2010). Conversely, there are emerging data that presence and extent of CIN are associated with inferior outcomes (Walther *et al*, 2008; Mouradov *et al*, 2013). Mutation in *BRAF* has been reported as an indicator of poor prognosis, in particular for patients with metastatic disease, but this relationship is complex, because of the strong positive association between *BRAF* mutation and MSI (Samowitz *et al*, 2005; French *et al*, 2008; Tie *et al*, 2011; Lochhead *et al*, 2013). Accordingly, recent data suggest that *BRAF* or *KRAS* mutation are associated with poor prognosis specifically in patients with MSS cancers (Phipps *et al*, 2015; Sinicrope *et al*, 2015). Evidence for the prognostic values of CIMP, *PIK3CA* and *TP53* mutation is inconsistent (Munro *et al*, 2005; Russo *et al*, 2005; Prenen *et al*, 2010; Day *et al*, 2013).

Although mutations in *APC* have a principal role in CRC initiation, their relation to outcome remains unclear. Most previous studies have not found an association between the presence of *APC* mutation and prognosis, but these analysed small patient cohorts, only screened limited regions of the *APC* gene, and did not account for prognostically important features such MSI, CIN, *BRAF* mutation and tumour location (Dix *et al*, 1994; Løvig *et al*, 2002; Conlin *et al*, 2005; Hsieh *et al*, 2005; Meguid *et al*, 2008; Chen *et al*, 2009; Wong, 2010; Birnbaum *et al*, 2012)

Evaluation of *APC* prognostic value is further complicated by the observation that proximal and distal tumours differ substantially in their *APC* mutation spectra. In general, somatic *APC* mutations tend to cluster in codons 1282–1581, the so-called mutation cluster region, producing truncated proteins retaining 1–3 intact 20 amino-acid repeats (20AARs), functional domains that are critical for β-catenin regulation (Miyoshi *et al*, 1992; Christie *et al*, 2013). However, when analysed by tumour location, proximal cancers show a marked enrichment for mutations leaving 2–3 20AARs, while distal cancers show predominance of mutations leaving 0–1 20AARs, indicating distinct WNT/β-catenin signalling thresholds for tumourigenesis in these embryologically distinct regions (Rowan *et al*, 2000; Albuquerque *et al*, 2002; Christie *et al*, 2013). A single prognostic study considered location of *APC* mutation and suggested that patients who have lost all β-catenin binding sites (15- and 20-AARs) of *APC* may have shorter cancer-related survival than patients with mutations that have retained one or more binding sites (Løvig *et al*, 2002); however, this study did not consider tumour location.

Here, we examined whether *APC* mutation presence or genotype are indicators of patient prognosis when accounting for tumour location, MSI, CIN, CIMP, *KRAS*, *BRAF*, *PIK3CA* and *TP53* status, analysing the largest CRC cohort ($n = 746$) to date in which the entire coding region of *APC* has been sequenced. We then identified the gene expression signature of *APC* mutation status using microarray analysis that could predict disease outcome. We validated the prognostic value of the *APC* classifier using an independent cohort of 206 patients from a publicly available microarray dataset (GSE39582) (Marisa *et al*, 2013) (Supplementary Figure 1).

MATERIALS AND METHODS

Patients. We analysed 746 patients with stages I-IV CRC who had undergone treatment at the Royal Melbourne Hospital (Parkville, VIC, Australia), Western Hospital Footscray (Footscray, VIC, Australia), Royal Adelaide Hospital (Adelaide, SA, Australia) and St Vincent's Hospital Sydney (Darlinghurst, NSW, Australia). Patients with familial polyposis syndromes, ulcerative colitis or Crohn's disease-associated CRC were excluded. All patients gave informed consent, and this study was human research ethics committee-approved (WEHI HREC 12/19). Clinicopathological characteristics and pre- and post-operative treatment details were collected using a multi-site database. Tumours from caecum to transverse colon were defined as proximal, and those from splenic flexure to rectum as distal. For patients with early stage disease (I-III), patient follow-up data were collected prospectively according to national guidelines, with 3-monthly clinic visits and testing for carcinoembryonic antigen levels, 12-monthly computed tomography scans of the chest, abdomen and pelvis for 2 years after diagnosis, and then 6-monthly clinic visits and carcinoembryonic antigen testing until 5 years from diagnosis. For patients with stage IV cancer, standard follow-up was with imaging every 8–9 weeks while patients remained on active therapy. Clinical follow-up while on active therapy was on a 4-weekly basis.

Mutation detection. Mutations in *APC* (entire coding region), *KRAS* (codons 12, 13 and 61), *BRAF* (V600E), *PIK3CA* (exons 9 and 20) and *TP53* (exons 4 to 9) have been determined previously (Christie *et al*, 2013; Day *et al*, 2013). Briefly, DNA was extracted from macrodissected tumour and matched normal tissues, and Sanger sequencing performed in both orientations on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Any detected mutations were confirmed by resequencing of tumour and matched normal DNA from new PCR product.

Microsatellite instability, CIN and loss of heterozygosity assessment. Microsatellite instability status was defined using the Bethesda five-marker microsatellite panel (Boland *et al*, 1998). Microsatellite instability was considered present if instability was seen at $\geqslant 2$ markers. Tumour CIN status and loss of heterozygosity

at *APC* have been determined previously using single nucleotide polymorphism microarray analysis (Human 610-Quad BeadChip, Illumina, San Diego, CA, USA) on tumour and matched normal DNA samples and OncoSNP software (Isis Innovations, Oxford, UK) (Yau *et al*, 2010; Christie *et al*, 2013; Mouradov *et al*, 2013).

CpG island methylator phenotype analysis. Tumour CIMP data have been reported based on MethyLight real-time PCR for the *IGF2*, *SOCS1*, *RUNX3*, *CACNA1G* and *NGN1* marker panel, and the reference ALU (Day *et al*, 2013). Tumours with a percentage of methylated reference value of greater than 10 for $\geqslant 3$ CIMP markers were classified as CIMP-high (CIMP-H), those with 1–2 methylated markers as CIMP-low (CIMP-L), and 0 methylated markers as CIMP-0.

Microarray analysis. Gene expression profiles were determined for 52 MSS proximal colon cancers using the Affymetrix (Santa Clara, CA, USA) GeneChip Human Exon 1.0 ST Array version 2 according to the manufacturer recommendations. The microarray data have been deposited in the Gene Expression Omnibus database (GSE63624). Data were normalised using the Robust Multi-array Average algorithm (Irizarry *et al*, 2003) from the Affymetrix Power Tools software, the normalised data were log transformed (base 2) and adjusted for a batch effect using the ComBat algorithm (Johnson *et al*, 2007). Probe sets that were not expressed or probe sets that showed a low variability across samples were excluded. Expression values were required to be greater than the median of all expression measurements in at least 25% of samples, with an interquartile range across samples on the log2 scale of greater than 0.5. Genes mapping to sex chromosomes were excluded because cases were not matched by gender. In addition, only probes for genes that were also represented on the Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays were considered to enable cross-referencing across these platforms.

Gene signature for *APC* mutation status. The Limma algorithm (Smyth, 2004) was used to rank gene probes associated with *APC*-wt status in our set of 52 MSS proximal colon cancers, with the top-ranked probe for each gene retained for classifier construction. We then identified the gene signature that could best classify *APC*-wt status using a linear kernel nu-support vector machine (SVM) algorithm (Chang and Lin, 2001) and 10-fold cross-validation, evaluating sets of 2–100 gene probes, selecting equal numbers of Limma top-ranked upregulated and down-regulated candidates. The expression data were mean-centred and scaled to a mean of zero and a standard deviation of one for each gene probe entered into the algorithm. The significance of classification accuracy was evaluated using permutation testing. First, the accuracy of the optimised discriminating classifier was measured by 500 times repeated 10-fold cross-validation. Then, class labels of the samples were permutated 10 000 times, obtaining a new signature and calculating the 10-fold cross-validation accuracy for each permutated data set. Finally, the random chance of obtaining a signature with higher accuracy than the optimised discriminating classifier was determined.

Validation of prognostic capability of *APC* gene signature in an independent dataset. We validated the prognostic capability of the *APC* gene signature using a publicly available independent microarray dataset on stages I to IV CRCs (GSE39582) (Marisa *et al*, 2013). Raw data were Robust Multi-array Average - normalised and batch-corrected using the ComBat algorithm, and restricted to proximal tumours with available outcome data ($n = 206$). For genes with multiple alternative probes, those with the largest variance across samples were selected. Gene probe data were mean-centred and scaled, and the SVM model used to predict *APC*-wt status.

Statistical analysis. Statistical analyses were conducted using the statistical computing software R (The R Development Core Team, 2013). Differences between groups were assessed using the Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables. Odds ratios (ORs) and 95% confidence intervals (CI) were obtained from Fisher's exact test calculations. Outcome analyses were performed for overall survival (OS) and recurrence-free survival (RFS) from date of surgery, censored at 5 years. Patients who had received radiotherapy or were stage IV and had undergone additional surgery for metastases were excluded. Univariate survival distributions were compared using the log-rank test. Cox proportional hazards models were used to estimate survival distributions and hazard ratios for multivariate analyses, adjusting for gender, age at diagnosis, tumour stage and treatment; to facilitate comparisons between multiple *APC*-wt/-mt tumour groups, hazard ratios were retrieved for all pairwise combinations of reference states. Comparisons between models with and without inclusion of specific molecular variables were made using the likelihood ratio test and the Aikake Information Criterion. Complete case analysis was used for all statistical calculations. Statistical analyses were two-sided and considered significant if $P < 0.05$.

RESULTS

Clinicopathological and molecular features of CRC patients. In the cohort of 746 patients, the median age at presentation was 70 years and 55% were male (Table 1). Seventy cancers were stage I, 229 stage II, 347 stage III and 100 stage IV; 315 were from the proximal colon, 242 from the distal colon and 189 from the rectum. Clinical follow-up information was available for 685 patients for OS. For patients with stages I-III CRC, RFS data were available for 415 cases. The median duration of follow-up was 47 months for OS, and 37 months for RFS. Among the 685 patients with available outcome data, 296 had received standard adjuvant 5-fluorouracil-based chemotherapy (unknown in 33 cases).

The cancers had molecular features that were similar to those found in other studies (Table 1). Truncating *APC* mutations were detected in 68.4% (510 of 746) of tumours, and 31.1% (201 of 627 with available single nucleotide polymorphism array data) showed loss of heterozygosity at *APC*. Among the *APC*-mt cases, 76.7% (335 of 437) had two hits (2 mutations or 1 mutation and loss of heterozygosity). The frequencies of mutations in *BRAF*, *KRAS*, *PIK3CA* and *TP53* were 9.0% (67 of 746), 35.1% (262 of 746), 12.5% (93 of 746) and 55.4% (413 of 746), respectively; 73.6% (465 of 632) of tumours were CIN, 13.4% (100 of 746) MSI, 17.3% (85 of 491) CIMP-H and 21.8% (107 of 491) CIMP-L. We confirmed the established pairwise associations between MSI, CIN and specific gene mutations. Microsatellite instability displayed a strong direct association with *BRAF* mutation (OR = 13.0), and a strong inverse association with *TP53* mutation (OR = 0.34), while CIN showed the opposite associations (CIN/*BRAF* OR = 0.20; CIN/*TP53* OR = 7.8). *KRAS* and *BRAF* mutations were mutually exclusive (OR = 0), while *KRAS* mutation exhibited positive associations with *PIK3CA* mutation (OR = 2.4) and CIMP-L (OR = 1.8 as compared with CIMP-0) ($P < 0.012$ for all comparisons).

Proximal *APC*-wt/MSS tumours exhibit inferior prognosis. We tested whether overall *APC* mutation status (wild-type *vs* mutated) was associated with patient prognosis when accounting for tumour MSI status and location, and adjusting for gender, age at diagnosis, tumour stage and treatment. Among patients with MSS tumours, *APC*-wt proximal cancers showed significantly inferior prognosis as compared with *APC*-mt proximal, *APC*-wt distal and *APC*-mt distal cancers for OS and RFS (OS HR $\geqslant 1.79$, $P \leqslant 0.015$; RFS HR $\geqslant 1.88$, $P \leqslant 0.026$) (Figure 1, Table 2A), and this was retained

Table 1. Characteristics of 746 patients with colorectal cancer according to *APC* mutation status

		APC-wt	*APC*-mt	
	***n* = 746**	**236 (31.6)**	**510 (68.4)**	***P***
Age, years	744	235	509	
Mean ± s.d.	69.1 ± 11.3	69.6 ± 11.7	68.9 ± 11.2	0.317
Median	70	70	70	
Range	25.0–99.0	25.0–99.0	33.0–93.0	
Unknown	2			
Sex	746	236	510	
Male	413 (55.4)	109 (26.4)	304 (73.6)	<0.001*
Female	333 (44.6)	127 (38.1)	206 (61.9)	
Stage	746	236	510	
I	70 (9.4)	15 (21.4)	55 (78.6)	0.080
II	229 (30.7)	67 (29.3)	162 (70.7)	
III	347 (46.5)	124 (35.7)	223 (64.3)	
IV	100 (13.4)	30 (30.0)	70 (70.0)	
Site	746	236	510	
Right Colon	315 (42.2)	131 (41.6)	184 (58.4)	<0.001*
Left colon	242 (32.4)	51 (21.1)	191 (78.9)	
Rectum	189 (25.3)	54 (28.6)	135 (71.4)	
Differentiation	718	221	497	
Well/ Moderate	596 (83.0)	162 (27.2)	434 (72.8)	<0.001*
Poor	122 (17.0)	59 (48.4)	63 (51.6)	
Unknown	28	15	13	
Mucinous	737	231	506	
No	577 (78.3)	154 (26.7)	423 (73.3)	<0.001*
Yes	160 (21.7)	77 (48.1)	83 (51.9)	
Unknown	9	5	4	
MSI status	746	236	510	
MSS	646 (86.6)	167 (25.9)	479 (74.1)	<0.001*
MSI	100 (13.4)	69 (69.0)	31 (31.0)	
CIMP status	491	140	351	
CIMP0	299 (60.9)	55 (18.4)	244 (81.6)	<0.001*
CIMPL	107 (21.8)	25 (23.4)	82 (76.6)	
CIMPH	85 (17.3)	60 (70.6)	25 (29.4)	
Unknown	255	96	159	
BRAF	746	236	510	
No	679 (91.0)	183 (27.0)	496 (73.0)	<0.001*
Yes	67 (9.0)	53 (79.1)	14 (20.9)	
KRAS	746	236	510	
No	484 (64.9)	185 (38.2)	299 (61.8)	<0.001*
Yes	262 (35.1)	51 (19.5)	211 (80.5)	
PIK3CA	746	236	510	
No	653 (87.5)	214 (32.8)	439 (67.2)	0.095
Yes	93 (12.5)	22 (23.7)	71 (76.3)	
TP53	746	236	510	
No	333 (44.6)	143 (42.9)	190 (57.1)	<0.001*
Yes	413 (55.4)	93 (22.5)	320 (77.5)	
CIN status	632	191	441	
CIN −	167 (26.4)	86 (51.5)	81 (48.5)	<0.001*
CIN +	465 (73.6)	105 (22.6)	360 (77.4)	
Unknown	114	45	69	

Abbreviations: CIN = chromosomal instability; CIMP = CpG island methylator phenotype; MSI = microsatellite instability; MSS = microsatellite stable. Row percentages are given in parentheses and column percentages are given in square brackets. *P<0.05.

when restricted to stage II and III cancers (OS HR⩾1.79, P⩽0.029; RFS HR⩾1.81, P⩽0.038) (Supplementary Table 1A). Accordingly, a multivariate model with an *APC*-tumour location interaction term provided a significantly better fit of the survival data as compared with a model with *APC* status and tumour location but without the interaction term (OS, P = 0.043; RFS, P = 0.011, likelihood ratio test). We found no evidence for differential outcomes between *APC*-mt/MSS proximal, *APC*-wt/MSS distal and *APC*-mt/MSS distal tumours (Figure 1, Table 2A, Supplementary Table 1A for stage II and III cancers).

No prognostic value of *APC* mutation in MSI tumours. Among patients with MSI cancers, testing for association between *APC* mutation status and prognosis was restricted to proximal cases owing to the low prevalence of MSI in distal tumours (n = 16). In contrast to MSS proximal cancers, the adverse prognostic influence of *APC*-wt status was lost in MSI proximal cancers (n = 75) with similar OS and RFS for wild-type and mutated tumours (multivariate OS, P = 0.859; RFS, P = 0.779, Figure 1, Table 2B, Supplementary Table 1B for stage II and III cancers).

Compared with MSI proximal cancers, *APC*-wt/MSS proximal tumours showed significantly poorer survival (OS, HR = 2.05, P = 0.022; RFS, HR = 3.34, P = 0.005). Microsatellite instability proximal tumours further exhibited a trend to improved outcomes as compared with *APC*-mt/MSS proximal cancers, although this was not statistically significant (OS, HR = 0.88, P = 0.656; RFS, HR = 0.59, P = 0.185) (Figure 2A, Table 3A). These trends were retained when restricting the analysis to stage II and III cancers (Supplementary Table 2A).

***APC* mutation genotype does not provide additional prognostic information.** Given the different *APC* mutation spectra in proximal and distal tumours (Albuquerque *et al*, 2010; Christie *et al*, 2013), we examined whether classification of tumours by *APC* mutation genotype had additional prognostic value. Among patients with *APC*-mt/MSS cancers, outcomes were similar across tumour locations irrespective of whether truncating *APC* mutations left 0 or ⩾1 15AARs required for β-catenin binding, or whether the mutations left 0-1 or 2-3 20AARs associated with residual β-catenin binding/regulatory activity (P⩾0.169 for all comparisons, Supplementary Table 3A and B). Further, no outcome differences were apparent when considering the number of hits in *APC* with one exception: proximal tumours with 1 hit in *APC* showed a tendency to better OS as compared with distal tumours with 1 hit in *APC* (HR = 0.37, P = 0.049), however, this trend was not observed for RFS (P = 0.246; Supplementary Table 3C). We did not explore *APC* genotypes in MSI CRCs owing to low sample numbers per group.

***APC* prognostic value in the context of other molecular changes.** The prognostic value of *APC* status in proximal MSS tumours was evaluated against other molecular changes. Single-variable models with *BRAF*, CIN, CIMP-H, *KRAS*, *PIK3CA* or *TP53* status were compared with models with addition of *APC* status and *vice versa* using the likelihood ratio test. In all comparisons, *APC* mutation was found to be the strongest indicator of outcome: addition of *APC* status significantly improved all OS and RFS models with CIMP, *BRAF*, *KRAS*, *PIK3CA* or *TP53* or CIN status (P⩽0.036 for all comparisons), whereas addition of the latter molecular variables to models with *APC* status did not significantly improve model fit (Table 4). Accordingly, direct comparison of models with either *APC* mutation or the relevant molecular feature of interest using the Akaike Information Criterion favoured the model with the *APC* mutation in all cases (Table 4).

***APC* gene signature in MSS proximal tumours.** For 52 patients with MSS proximal colon cancer, including 35 *APC*-mt patients, we were able to obtain sufficient tumour RNA of high quality for

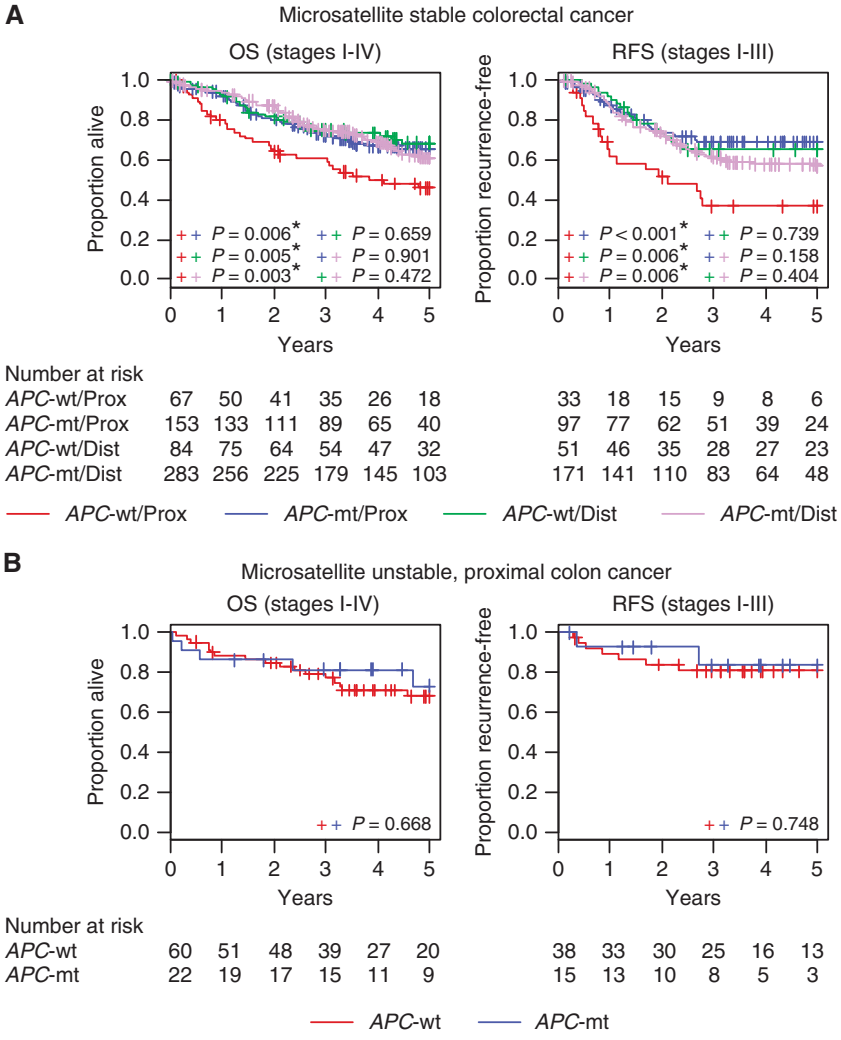

Figure 1. Kaplan-Meier curves of OS and RFS (**A**) for patients with MSS colorectal cancer, and (**B**) for patients with MSI proximal colon cancer, according to *APC* mutation status and tumour location. Abbreviations: Dist, distal; Prox, proximal.

microarray analysis. Using class-comparison analysis, we ranked genes by significance of differential expression between *APC*-wt and *APC*-mt patients. A SVM with 10-fold cross-validation was used to identify the gene signature that could best classify *APC*-wt status, with a classifier comprising the top-ranked 18 upregulated and 18 downregulated gene probes achieving the highest cross-validation prediction accuracy (95.8%) (Supplementary Figure 2, Supplementary Table 4). Using these 36 discriminating genes, two-way clustering and principal component analysis separated *APC*-wt and *APC*-mt patients (Supplementary Figure 3). To further evaluate the SVM model, we re-trained the model using 10 000 random permutations of the *APC* mutation data. None of these permutations yielded equal or superior 10-fold cross-validation prediction accuracy to that of the trained model, yielding a false discovery rate less than 10^{-4}. *APC*-wt gene signature tumours exhibited statistically significantly worse survival than *APC*-mt gene signature tumours, as was observed when considering *APC* mutation status determined by Sanger sequencing (Supplementary Figure 4).

Validation of prognostic capability of *APC*-wt gene signature in an independent cohort of proximal colon cancers. We applied the *APC*-wt gene signature to an independent microarray dataset on 206 stage I-IV proximal colon cancers by Marisa *et al* (2013) (GSE39582). Consistent with findings in our cohort, patients with *APC*-wt-like gene signature MSS tumours showed significantly poorer OS and RFS than those with *APC*-mt-like gene signature MSS tumours and MSI tumours (OS HR$\geqslant$2.50, $P\leqslant$0.010; RFS HR$\geqslant$2.14, $P\leqslant$0.025) (Figure 2B, Table 3B, Supplementary Table 2B when restricting to stage II and III cancers). There was no significant difference in outcome by *APC* class for MSI cancers ($P\geqslant$0.375 for OS and RFS).

Proximal *APC*-wt/MSS tumours exhibit features of the sessile serrated pathway. Given the differences in prognosis among MSS CRCs by *APC* status and tumour location, we tested whether these were reflected at the pathological and molecular level. In pairwise comparisons between tumour groups (Figure 3, Supplementary Table 5), the poor prognosis *APC*-wt/MSS cancers of the proximal colon ($n=70$) consistently showed associations with features of the sessile serrated pathway including poor differentiation, CIMP-H and *BRAF* mutation ($P\leqslant$0.016 for all comparisons), and to a lesser extent mucinous histology ($P\leqslant$0.058) and female gender ($P\leqslant$0.085, Figure 3A). *APC*-mt/MSS distal cancers ($n=318$) displayed the expected classic adenoma-carcinoma pathway features such as *TP53* mutation and CIN ($P\leqslant$0.020 for all comparisons, Figure 3D), while *APC*-mt/MSS proximal cancers ($n=161$) showed association with *KRAS* mutation ($P<$0.001 for all comparisons) and to a lesser extent with *PIK3CA* mutation ($P\leqslant$0.054 for all comparisons), hallmarks of the alternate pathway (Figure 3B). *APC*-wt/MSS distal cancers ($n=97$) showed no consistently outstanding characteristics, although some tendency

Table 2A. Cox proportional-hazards analyses of OS and RFS (A) for patients with microsatellite stable (MSS) colorectal cancer, and (B) for patients with microsatellite unstable (MS) proximal colon cancer, according to *APC* mutation status and tumour location; (2A) Microsatellite stable (MSS) colorectal cancer

	Overall survival		Recurrence-free survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
APC-wt/Prox *vs* *APC*-mt/Prox	1.79 (1.12–2.85)	0.015*	1.99 (1.08–3.65)	0.026*
APC-wt/Prox *vs* *APC*-wt/Dist	2.01 (1.17–3.43)	0.011*	2.71 (1.39–5.28)	0.003*
APC-wt/Prox *vs* *APC*-mt/Dist	1.84 (1.22–2.78)	0.004*	1.88 (1.13–3.15)	0.016*
APC-mt/Prox *vs* *APC*-wt/Dist	1.12 (0.67–1.87)	0.655	1.36 (0.73–2.54)	0.329
APC-mt/Prox *vs* *APC*-mt/Dist	1.03 (0.71–1.49)	0.876	0.95 (0.60–1.50)	0.814
APC-wt/Dist *vs* *APC*-mt/Dist	0.92 (0.58–1.45)	0.710	0.69 (0.40–1.19)	0.186
Age (Decades)	1.32 (1.12–1.55)	<0.001*	0.93 (0.79–1.09)	0.386
Gender (Female *vs* Male)	0.77 (0.57–1.04)	0.089	0.66 (0.46–0.95)	0.024*
Stage II *vs* I	1.03 (0.46–2.30)	0.938	1.88 (0.54–6.50)	0.320
Stage III *vs* I	3.33 (1.56–7.10)	0.002*	9.23 (2.78–30.65)	<0.001*
Stage IV *vs* I	9.62 (4.33–21.36)	<0.001*		
Chemotherapy (Yes *vs* No)	0.82 (0.57–1.17)	0.270	0.68 (0.43–1.09)	0.107
Events/*N*	189/561		129/351	

Abbreviations: CI = confidence interval; Dist = distal; HR = hazard ratio; OS = overall survival; Prox = proximal; RFS = recurrence-free survival. To facilitate comparisons between *APC* wild-type/mutated tumour groups, hazard ratios are presented for all pairwise combinations of reference states.

Table 2B. (B) Microsatellite unstable (MSI) proximal colon cancer

	Overall survival		Recurrence-free survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
APC-wt v *APC*-mt	0.90 (0.27–2.96)	0.859	1.26 (0.25–6.50)	0.779
Age (Decades)	2.37 (1.30–4.34)	0.005*	0.95 (0.40–2.25)	0.900
Gender (Female vs Male)	1.06 (0.31–3.59)	0.924	1.58 (0.32–7.80)	0.576
Stage III *vs* I/II[a]	0.95 (0.31–2.90)	0.933	3.36 (0.62–18.30)	0.161
Stage IV *vs* I/II[a]	5.54 (0.38–81.75)	0.213		
Chemotherapy (Yes *vs* No)	2.10 (0.47–9.37)	0.330	0.25 (0.03–1.99)	0.189
Events/*N*	17/75		9/53	

Abbreviations: CI = confidence interval; Dist = distal; HR = hazard ratio.
[a]Combined stages I/II referent owing to small sample number. *$P<0.05$.

towards features of the sessile serrated pathway was noted when compared with *APC*-mt/MSS distal cancers (mucinous histology, CIMP-H and *BRAF* mutation; $P \leqslant 0.009$ for all comparisons). However, these differences were much less pronounced than for MSS/*APC*-wt proximal cancers (Figure 3C).

In contrast, among good prognosis MSI proximal cancers ($n = 82$), for which the *APC* prognostic value was attenuated, no significant differences were apparent by *APC* status (Supplementary Table 6). When MSI proximal cancers were compared with the four groups of MSS cancers, these most closely resembled the poor prognosis *APC*-wt/MSS proximal group, exhibiting an overrepresentation of sessile serrated pathway features such as female gender, CIMP-H, *BRAF* mutation, poor differentiation and mucinous histology (Figure 3E, Supplementary Table 5). However, MSI proximal cancers differed from all four groups in showing little CIN and a tendency to present at earlier tumour stages. Associations for MSI distal cancers were not investigated owing to the small sample size ($n = 16$).

DISCUSSION

Prognostic, pathological and molecular features of CRC, including *APC* mutation spectra, are well-established to differ between proximal and distal tumours and between MSS and MSI cases (Meguid *et al*, 2008; Albuquerque *et al*, 2010; Wong, 2010; Sinicrope and Sargent, 2012; Christie *et al*, 2013). In this study, the largest survey of the prognostic value of *APC* mutation to date, we have found evidence that *APC* status shows differential outcome associations across these tumour subgroups: *APC*-wt/MSS proximal tumours had inferior OS and RFS as compared with *APC*-mt/MSS proximal, *APC*-wt/MSS distal and *APC*-mt/MSS distal tumours, which showed similar outcomes. The prognostic value of *APC* mutation was lost in the MSI tumours of the proximal colon, which had the most favourable prognosis overall. Notably, attenuation of marker prognostic value in MSI cancers has previously been reported for *BRAF* mutation and CIMP (Ward *et al*, 2003; Samowitz *et al*, 2005; Ogino *et al*, 2012). Poor prognosis of *APC*-wt/MSS proximal tumours was validated in an independent patient cohort (Marisa *et al*, 2013) using a predictor of *APC*-wt status from microarray expression data. Most previous studies evaluating *APC* mutation status have not identified a relationship with prognosis (Dix *et al*, 1994; Conlin *et al*, 2005; Hsieh *et al*, 2005; Chen *et al*, 2009; Birnbaum *et al*, 2012). However, these studies did not consider CRC groups by tumour location and MSI status, likely precluded by their smaller sample sizes ($n = 100$–218), and only performed mutation screening for limited regions of the *APC* gene. These differences in study design and size would have reduced the power to detect tumour-subgroup-specific outcome associations.

Differences in tumour prognostic behaviours by *APC* mutation, location and MSI status were strongly supported by differences at the pathological and molecular level (Supplementary Figure 5). The poor prognosis *APC*-wt/MSS proximal cancers were specifically associated with characteristics of the sessile serrated neoplasia pathway including female gender, poor differentiation, mucinous histology, CIMP-H and *BRAF* mutation (Leggett and Whitehall, 2010; Snover, 2011; Bettington *et al*, 2013, 2014). In contrast, *APC*-mt/MSS proximal cancers showed features of the alternate pathway (*KRAS* and *PIK3CA* mutation) (Leggett and Whitehall, 2010; Day *et al*, 2013). *APC*-mt/MSS distal cancers showed features of the classic adenoma-carcinoma pathway (*TP53* mutation and CIN) (Fearon and Vogelstein, 1990), whereas *APC*-wt/MSS distal cancers had no consistently outstanding features. CpG island methylator phenotype-L, which has been associated with the alternate pathway in some studies (Leggett and Whitehall, 2010), was not overrepresented in *APC*-mt/MSS proximal cancers, although a positive association between CIMP-L and *KRAS* mutation was apparent for the entire cohort.

Among MSI cancers of the proximal colon, for which the prognostic value of *APC* mutation was attenuated, pathological and molecular features accordingly also did not differ by *APC* status. Overall, MSI proximal cancers showed the expected serrated pathway features (female gender, poor differentiation, mucinous histology, CIMP-H and *BRAF* mutation), similar to the poor prognosis proximal *APC*-wt/MSS group, but differed in showing little CIN and presentation at earlier tumour stages.

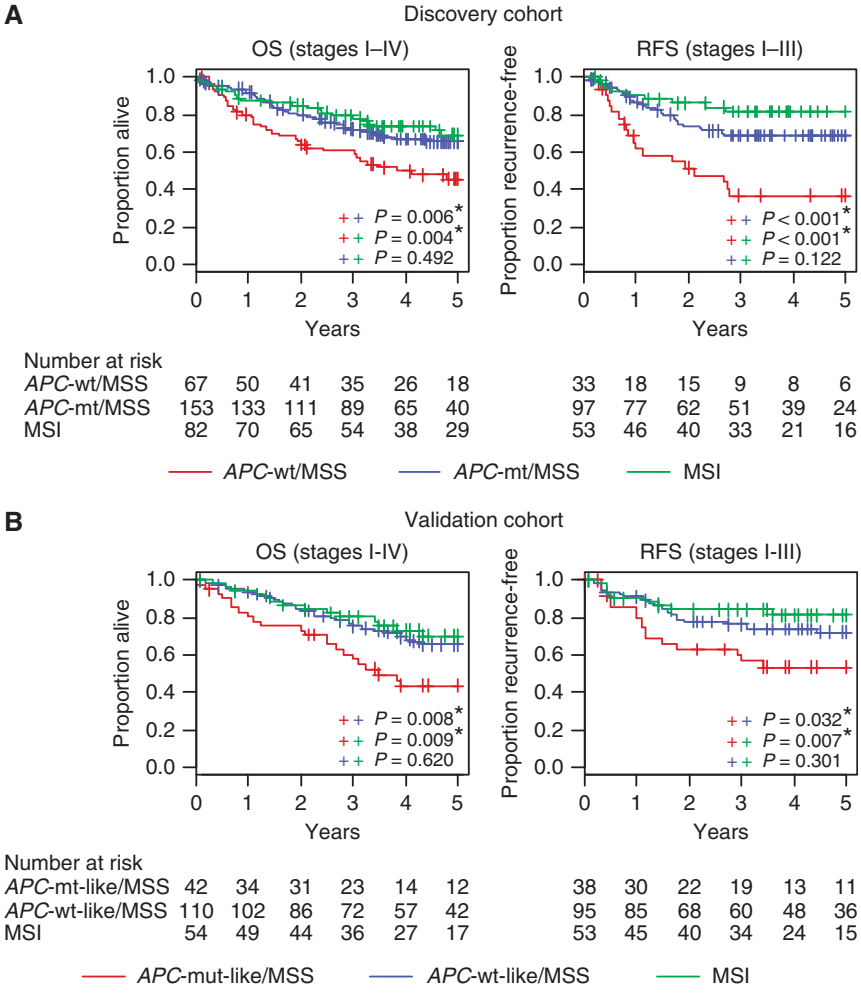

Figure 2. Kaplan-Meier curves of OS and RFS (**A**) for patients with proximal colon cancer, according to *APC* mutation and microsatellite instability status (discovery cohort), and (**B**) for patients with proximal colon cancer, according to *APC* gene signature and microsatellite instability status (validation cohort, GSE39582 (Marisa *et al*, 2013)). Abbreviations: MSS/MSI, microsatellite stable/unstable.

The prognostic value of *APC* mutation status in MSS proximal cancers was not explained by correlation with other tumour molecular features such as CIN, CIMP, *BRAF*, *KRAS*, *PIK3CA* or *TP53* status. This is particularly notable for CIN, *BRAF* and *KRAS* mutation, which have been linked to inferior prognosis in MSS tumours (Tie *et al*, 2011; Pai *et al*, 2012; Lochhead *et al*, 2013; Sinicrope *et al*, 2015). Instead, our data suggest a more general association between tumour sessile serrated pathway features and poor prognosis, which is supported by recent gene expression-based studies. Using microarray analysis to create a gene expression classifier for *BRAF*-mutated CRCs, Popovici *et al* (2012) identified a group of cancers including but not restricted to *BRAF*-mutated tumours which showed poor prognosis, proximal location and mucinous histology, consistent with serrated pathway characteristics. Similarly, De Sousa *et al* (2013) used multiple microarray datasets to identify a CRC subgroup displaying a sessile serrated adenoma-like gene expression signature and inferior prognosis as compared with cancers with MSI- or CIN-associated signatures. A recent molecular profiling study has further reported poor prognosis for MSS or MSI-low, CIMP-positive, *BRAF* mutated, *KRAS* wild-type CRCs, which likely have significant overlap with the *APC*-wt/MSS proximal subtype defined in our study (Phipps *et al*, 2015).

Methylation at the *APC* promoter resulting in gene silencing has been suggested as a potential alternative mechanism to *APC* mutation (Arnold *et al*, 2004). Using DNA methylation array and RNA-Seq data for 215 CRCs reported by The Cancer Genome Atlas Network, neither promoter hypermethylation nor reduced gene expression were inversely associated with *APC* mutation when considering all cases or the subset of MSS proximal cancers (Supplementary Figure 6) (Network TCGA, 2012). These results are consistent with our previous observation that *APC* promoter hypermethylation does not substitute for truncating mutations (Segditsas *et al*, 2008).

Interestingly, *AXIN2* and *RNF43* were the most differentially expressed genes by *APC* mutation status in proximal MSS tumours, with both showing reduced expression in the *APC*-wt tumours. AXIN2 is a component of the central β-catenin destruction complex, while RNF43 is a transmembrane E3 ligase involved in removing WNT receptors from the cell surface (Jho *et al*, 2002; de Lau *et al*, 2014). *AXIN2* and *RNF43* are themselves WNT target genes and constitute negative WNT feedback loops, suggesting that their downregulation may be an alternative to *APC* mutation causing aberrant WNT pathway activation. *AXIN2* and *RNF43* are frequently mutated in MSI CRCs (Liu *et al*, 2000; Giannakis *et al*, 2014), and *AXIN2* silencing by promoter hypermethylation has been observed in MSI cancers and sessile serrated adenomas (Muto *et al*, 2014). Furthermore, *AXIN2* and *RNF43* were found in the Popovici *et al* (2012) signature for *BRAF*-mutated CRC and the De Sousa *et al* (2013) gene signatures for CRC classification.

It has been suggested that *APC*-mutated cancers may show differential outcomes depending on mutation location within the

gene, with patients who have lost all β-catenin binding sites having shorter cancer-related survival than patients who have retained one or more binding sites (Løvig *et al*, 2002). We did not find evidence of outcome differences by the number of intact β-catenin binding sites or when comparing tumours with one or two detected hits. While the number of intact β-catenin binding sites appears to be important for tumour initiation, with different 'just-right' mutation spectra in the embryologically distinct proximal and distal colon (Albuquerque *et al*, 2010; Christie *et al*, 2013), *APC* mutation genotypes appear to have less influence on disease progression.

Although further large cohort studies will be required to validate our findings, our data suggest a potential role for joint MSI and *APC* testing in risk stratification for patients with CRC. In particular, more aggressive investigation and therapy may be indicated for patients with poor prognosis *APC*-wt/MSS proximal

Table 3A. Cox proportional-hazards analyses of OS and RFS (A) for patients with proximal colon cancer, according to *APC* mutation status and microsatellite instability (discovery cohort); and (B) for patients with proximal colon cancer, according to *APC* gene signature and microsatellite instability (validation cohort, GSE39582 (Marisa *et al*, 2013)); (A) Discovery cohort

	Overall survival		Recurrence-free survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
MSI *vs* *APC*-mt/MSS	0.88 (0.49–1.57)	0.656	0.59 (0.27–1.29)	0.185
APC-wt/MSS/*vs* MSI	2.05 (1.11–3.77)	0.022*	3.34 (1.45–7.68)	0.005*
APC-wt/MSS *vs* *APC*-mt/MSS	1.79 (1.11–2.88)	0.016*	1.97 (1.06–3.63)	0.031*
Age (Decades)	1.41 (1.09–1.82)	0.009*	1.08 (0.81–1.45)	0.588
Gender (Female *vs* Male)	0.92 (0.60–1.42)	0.718	0.93 (0.54–1.60)	0.794
Stage II *vs* I	1.85 (0.43–8.06)	0.412	1.19 (0.25–5.68)	0.824
Stage III *vs* I	5.04 (1.16–21.82)	0.030*	5.84 (1.26–27.04)	0.024*
Stage IV *vs* I	19.21 (4.25–86.76)	<0.001*		
Chemotherapy (Yes *vs* No)	1.03 (0.60–1.76)	0.919	0.91 (0.44–1.88)	0.807
Events/*N*	91/282		55/182	

Abbreviations: CI = confidence interval; HR = hazard ratio; MSI = microsatellite instability; MSS = microsatellite stable; OS = overall survival; RFS = recurrence-free survival. Analyses are adjusted for gender, age at diagnosis, tumour stage and treatment. To facilitate comparisons between *APC* wild-type/mutated tumour groups, hazard ratios are presented for all pairwise combinations of reference states.

Table 3B. (B) Validation cohort

	Overall survival		Recurrence-free survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
MSI *vs* *APC*-mt/MSS	1.21 (0.63–2.34)	0.569	0.70 (0.32–1.54)	0.375
APC-wt/MSS/*vs* MSI	2.50 (1.25–4.98)	0.010*	3.06 (1.34–6.98)	0.008*
APC-wt/MSS *vs* *APC*-mt/MSS	3.02 (1.67–5.47)	<0.001*	2.14 (1.10–4.18)	0.025*
Age (Decades)	1.45 (1.15–1.83)	0.002*	1.12 (0.87–1.44)	0.374
Gender (Female *vs* Male)	0.37 (0.22–0.63)	<0.001*	0.47 (0.26–0.85)	0.012*
Stage II *vs* I	0.74 (0.22–2.51)	0.630	2.23 (0.30–16.77)	0.437
Stage III *vs* I	1.17 (0.34–4.06)	0.809	4.21 (0.55–32.46)	0.167
Stage IV *vs* I	4.86 (1.22–19.34)	0.025*		
Chemotherapy (Yes *vs* No)	1.26 (0.68–2.32)	0.458	0.89 (0.43–1.82)	0.742
Events/*N*	65/201		49/185	

Abbreviation: CI = confidence interval; HR = hazard ratio; MSI = microsatellite instability; MSS = microsatellite stable. *$P<0.05$.

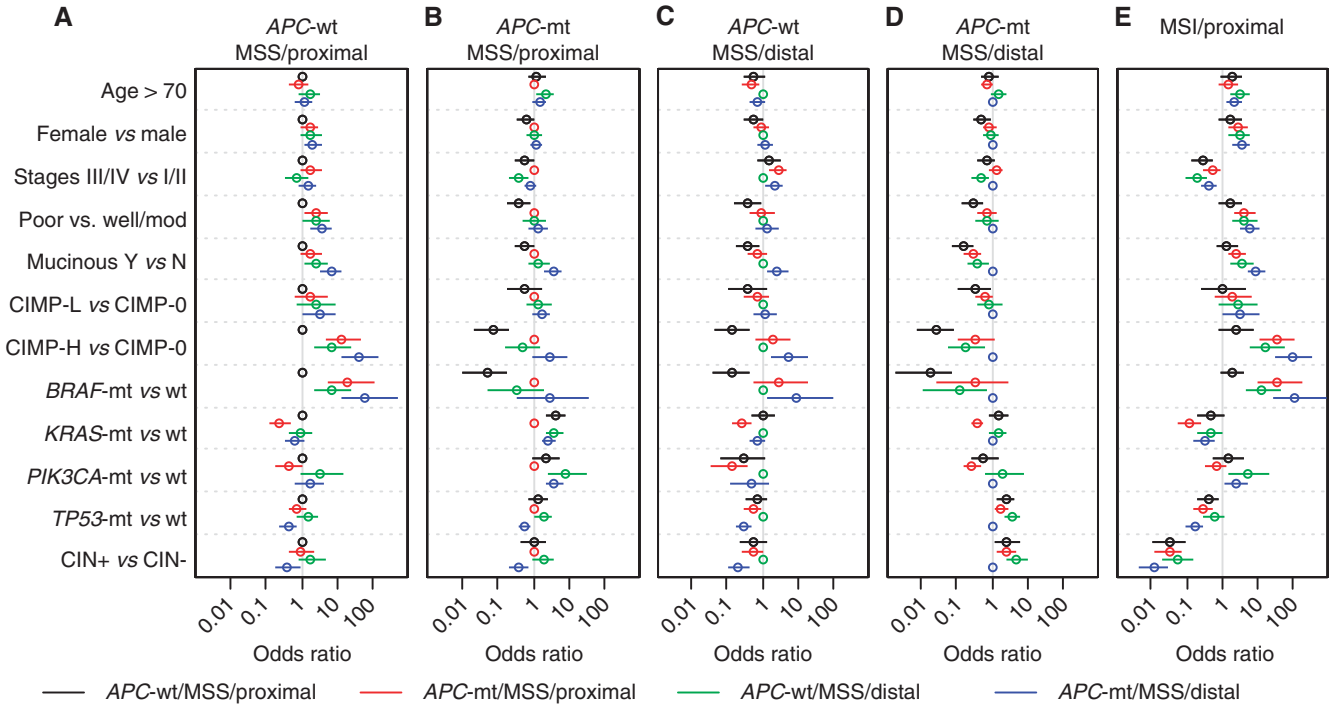

Figure 3. Comparison of clinicopathological and molecular characteristics between colorectal cancer groups defined by *APC* mutation status, microsatellite instability and tumour location. Odds ratios (circles) and 95% confidence intervals (lines) for (**A**) *APC*-wt/MSS proximal cancers, (**B**) *APC*-mt/MSS proximal cancers, (**C**) *APC*-wt/MSS distal cancers, (**D**) *APC*-mt/MSS distal cancers, relative to each other. (**E**) Comparison of MSI proximal cancers to the four MSS tumour groups. Abbreviations: CIN chromosomal instability; CIMP, CpG island methylator phenotype; MSS/MSI, microsatellite stable/unstable.

Table 4. Likelihood ratio tests evaluating the fit of OS and RFS data for patients with microsatellite stable, proximal colon cancer when adding *APC* mutation status to Cox proportional-hazard models with CIMP-H, BRAF, KRAS, PIK3CA, TP53 or CIN status (labeled X) or vice versa

	Overall survival				Recurrence-free survival			
	Events/*N*	P (add *APC*)	P (add X)	AIC (*APC*)-AIC (X)	Events/N	P (add *APC*)	P (add X)	AIC (*APC*)-AIC (X)
CIMP status	58/149	0.003*	0.542	−9.3	43/123	0.021*	0.731	−6.7
BRAF-mt	79/220	0.036*	0.323	−3.4	46/130	0.014*	0.464	−5.6
KRAS-mt	79/220	0.012*	0.827	−6.3	46/130	0.002*	0.470	−9.4
PIK3CA-mt	79/220	0.010*	0.181	−4.8	46/130	0.002*	0.487	−8.8
TP53-mt	79/220	0.007*	0.403	−6.7	46/130	0.001*	0.178	−8.4
CIN status	71/194	<0.001*	0.904	−11.2	44/123	0.001*	0.096	−8.1

Abbreviations: CIMP = CpG island methylator phenotype; CIN = chromosomal instability; MSS = microsatellite stable; OS = overall survival; RFS = recurrence-free survival. *$P<0.05$. Differences in Aikake Information Criteria (AIC) are shown for the comparison of models with *APC* mutation against the relevant molecular feature of interest; negative values of the AIC difference indicate a better fit for the *APC* mutation models.

tumours. As *APC* is a large gene with mutations occurring throughout the 5' two-thirds of the coding sequence, demonstration of *APC*-wt status will require substantial sequencing, which would previously have been impractical for clinical purposes. However, with the increasing adoption of massively parallel sequencing in clinical molecular pathology departments, routine *APC* sequencing for prognostic purposes will become feasible.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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